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Analytical Methods

An effective and simple procedure to isolate abundant quantities of biologically active chemopreventive Lunasin Protease Inhibitor Concentrate (LPIC) from soybean

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ABSTRACT

Lunasin is a 5-kDa soybean bioactive peptide with demonstrated anti-cancer and anti-inflammatory properties. Recently, purification methods have been developed to obtain gram quantities of lunasin. However, these methods are cumbersome, time consuming and cost-prohibitive. To overcome these constrains we have developed a novel method which involves extraction of soybean flour with 30% ethanol followed by preferential precipitation of lunasin and protease inhibitors by calcium. The calcium precipitated protein fraction, which we termed as Lunasin Protease Inhibitor Concentrate (LPIC), contains three abundant proteins with molecular weights of 21, 14 and 5 kDa. This simple procedure yields 3.2 g of LPIC from 100 g of soybean flour and the entire isolation procedure can be completed in less than 2 h. Treatment of THP-1 human monocyte cell lines with LPIC resulted in suppression of lipopolysaccharide-stimulated cytokine expression, demonstrating that the LPIC isolated by our simple procedure is biologically active.

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1. Introduction

Breast cancer is the second most leading cause of death among American women (http://www.cdc.gov/cancer/dcpc/data/women.htm). Even though breast cancer is prevalent in the rest of the world, the incidence of breast cancer is significantly lower among women in Asian countries when compared to women in Western countries (Messina, Nagata, & Wu, 2006; Wu, Yu, Tseng, & Pike, 2008). About a 6-fold difference in breast cancer risk has been reported between women from these two groups and additional studies have shown that this difference is not due to genetic factors (Korde et al., 2009). One contributing factor for the low incidence of breast cancer in Asian women may be attributed to their soy-rich diet (Messina & Loprinzi, 2001). Soy has been consumed in Asian countries for at least the last 5000 years and it is believed to offer health benefits (Messina & Loprinzi, 2001; Messina & Messina, 1991).

Scientific studies have shown that consumption of soy/soy products may reduce the risk for breast, colon and prostate cancer (Badger, Ronis, Simmen, & Simmen, 2005). Interestingly, it has been reported that women who regularly consumed soy/soy products were 25% less likely to have their cancer return (Nechuta et al., 2012). Even though isoflavones have been implicated as a major player in reducing the risk of cancer, other studies have shown a pivotal role for soy protease inhibitors as putative chemopreventive agents (Armstrong, Wan, Kennedy, Taylor, & Meyskens, 2003; Armstrong et al., 2000; Kennedy, 1998; Kennedy & Wan, 2002; Yavelow, Collins, Birk, Troll, & Kennedy, 1985). Soy contains two types of protease inhibitors, the Kunitz and Bowman-Birk inhibitors (Krishnan, 2000; Nielsen, 1996). Bowman-Birk inhibitor (BBI) is a double-head serine protease inhibitor that can inhibit both trypsin and chymotrypsin (Birk, 1985). This protein has been shown to be very effective in suppressing carcinogenesis (Kennedy, 1998). Since purification of large quantities BBI is cost prohibitive, Kennedy and her associates developed a soybean extract that was concentrated in BBI (Kennedy, Szuhaj, Newberne, & Billings, 1993). This extract, which was termed BBI concentrate (BBIC),

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was found to be effective as the purified BBI as an anti-carcinogenic agent (Kennedy, 1998).

It was assumed that the active anticarcinogenic component in BBIC was solely BBI, a potent chymotrypsin inhibitor (Kennedy, 1998). However, a recent study has shown that in addition to BBI, other protein components present in the BBIC may also contribute to the chemopreventive function of BBIC (Hsieh, Hernández-Ledesma, Jeong, Park, & de Lumen, 2010). Biochemical analyses have revealed that BBI and lunasin are the two predominant protein components of BBIC, representing about 44% of the total protein of BBIC (Hsieh et al., 2010). Lunasin, a 43-amino acid peptide derived from soy, has been shown to prevent malignant transformation and carcinogenesis *in vitro* (de Lumen, 2005, 2008). It was proposed that lunasin, not BBI, as the main chemopreventive agent in BBIC and the role of BBI was to protect the lunasin from being digested by humans (Hsieh et al., 2010). Extensive research has now established the pivotal role of lunasin as a chemopreventive agent along with an important function in inhibiting inflammation associated with chronic diseases (Cam & de Mejia, 2012; Dia & de Mejia, 2011a,b; Galvez & de Lumen, 1999).

In spite of its potential as a chemopreventive agent lunasin has not been widely used in large-scale animal and human clinical studies. A prerequisite for conducting such studies is the availability of large quantities of purified lunasin. All soybean varieties that have been examined contain lunasin and their concentration ranged from 0.5 to 8.1 mg lunasin per gram of seed (de Mejia, Vasconez, de Lumen, & Nelson, 2004; Wang, Dia, Vasconez, Nelson, & de Mejia, 2008). Attempts have been made to isolate and purify lunasin in gram quantities (Cavazos, Morales, Dia, & de Mejia, 2012; Dia, Wang, Oh, de Lumen, & de Mejia, 2009; Seber et al., 2012). One approach was by expressing lunasin in *Escherichia coli* or in the yeast *Pichia pastoris* (Lin, Fido, Archer, & Alcocer, 2004; Liu & Pan, 2010). Seber and his associates (2012) were able to isolate 442 mg lunasin from a kilogram of defatted soy flour by employing anion-exchange chromatography, ultrafiltration, and reversed-phase chromatography. However these studies are cost prohibitive, cumbersome and involve time-consuming analytical instruments that will pose limitations on large-scale purification of lunasin. In order to overcome such limitations we have developed an alternative strategy that focuses on isolation of soy protein extract that is highly enriched in lunasin and protease inhibitors, proteins with well-established roles in cancer prevention. This extract, which we have coined as Lunasin Protease Inhibitor Concentrate (LPIC) was effective in suppressing lipopolysaccharide-stimulated cytokine expression in THP-1 human monocyte cell lines.

2. Materials and methods

2.1. Reagents

Acrylamide, bis-acrylamide, ammonium persulfate, TEMED, Coomassie Brilliant Blue R-250, and Goat antirabbit IgG–horseradish peroxidase (HRP) conjugate were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Calcium chloride dihydrate was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Cell culture media, DMEM and RPMI 1,640 were obtained from GIBCO (Grand Island, NY, U.S.A.). DE52 Pre-Swollen Microgranular DEAE Anion Exchange Cellulose and lipopolysaccharides from *E. coli* 0111:B4 were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, U.S.A.). StrataScript First Strand Complementary DNA Synthesis kit was obtained from Stratagene (Santa Clara, CA, U.S.A.). TaqMan Universal PCR Master Mix for Real-time PCR was from Applied Biosystems (Carlsbad, CA, U.S.A.).

2.2. Chemical synthesis of lunasin peptide

Soy lunasin (SKWQHQQDSCRKQLQGVNLTPC-EKHIMEKIQGR-GDDDDDDDDD) was synthesized by United BioSystems Inc (Herndon, VA, U.S.A.). The lyophilized lunasin peptide was stored at −20 °C until used.

2.3. Lunasin peptide antibodies

A 20 amino acid peptide (SKWQHQQDSCRKQLQGVNLT) and a 15 amino acid peptide (CEKHIMEKIQGRGDD) corresponding to the N-terminal and C-terminal of the mature protein respectively, was synthesized and antibodies to these peptides were raised in rabbits by Proteintech Group Inc. (Chicago, IL, U.S.A.). The antibodies were stored in small aliquots at −80 °C until used.

2.4. Lunasin enrichment

For small-scale lunasin isolation, 100 mg of finely ground soybean cv. Williams 82 seed powder was transferred to a 2 mL Eppendorff tube and extracted with 1 mL of 30% ethanol on a 37 °C shaker for 30 min. The slurry was subjected to centrifugation at 15,800×*g* for 10 min and the resulting supernatant was saved. An aliquot (200 μL) of the clear supernatant was transferred to a new tube and 20 μL of 100 mM calcium chloride was added. The contents of the tube were thoroughly mixed and left on the bench top for 5 min. This was followed by centrifugation at 15,800×*g* for 10 min. The resulting pellet was briefly air-dried, resuspended in 200 μL of 1× SDS-sample treatment buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 30 mM Bromphenol Blue, pH 6.8, 5% (v/v) β-mercaptoethanol) and heated in a boiling water bath for 5 min.

2.5. Large-Scale isolation of Lunasin Protease Inhibitor Concentrate (LPIC)

One hundred grams of finely ground soybean cv. Williams 82 seed was transferred to a 2.8 L Fernbach culture flask containing 1 L of 30% ethanol and placed on a reciprocal shaker (100 rpm) for 30 min. The slurry was transferred to large plastic centrifuge bottles and subjected to centrifugation at 10,000×*g* for 30 min at 4 °C. The clear supernatant was saved and transferred to a new flask. For enriching lunasin, a 1 M $CaCl_2$ stock solution was added with gentle stirring to bring the final concentration of calcium chloride to 10 mM. After through mixing, the solution was left to stand at room temperature for 5 min. The precipitated proteins were recovered by centrifugation as before. The supernatant from this step was discarded and the pellet was lyophilized. The resulting white material was ground to a fine powder with a mortar and pestle and named as LPIC.

2.6. DE-52 anion-exchange chromatography

Additional purification of LPIC was carried out by DE-52 anion exchange cellulose chromatography. Typically, lyophilized LPIC obtained from 100 g of soy seed powder was resuspended in 50 mL of 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and dialyzed overnight against the same buffer. The dialyzed LPIC was centrifuged at 10,000×*g* for 15 min at 4 °C to remove precipitated proteins. An aliquot (30 mL) was applied at a flow rate of 3 mL/min to a column of DE-52 (5 × 15.5 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA. The unbound proteins were washed with 2 column volumes of the same buffer and then subjected to a sequential elution with 50 mL of sodium phosphate buffer each with increasing concentrations (50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 500 mM and 1 M) of NaCl. The volume of each

fraction collected was 6 mL. The protein concentration was monitored by measuring the absorbance at 280 nm and every alternative fraction was analyzed by SDS–PAGE and Western blot analysis.

2.7. SDS–PAGE analysis

SDS–PAGE was performed using a Hoefer SE-260 minigel apparatus (Amersham Biosciences, Piscataway, NJ, U.S.A.) as previously described (Krishnan, Oehrle, & Natarajan, 2009). Prior to electrophoretic analysis, protein samples were heated in a boiling water bath for 5 min. Electrophoresis was conducted at 20 mA/gel for 1 h and separated proteins were visualized by staining the gel overnight with Coomassie Blue R-250.

2.8. Densitometric analysis

Computer-assisted analysis was performed as described earlier (Krishnan, Jang, Baxter, & Wiebold, 2012) by utilizing the Advanced Analysis (version 4.01) module of Phoretix 1D gel analysis software (Nonlinear Dynamics Limited, Newcastle, UK).

2.9. Western blot analysis

Soy proteins were first fractionated on a 15% SDS–PAGE gels. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher & Schuell Inc., Keene, NH). The effectiveness of the protein transfer was monitored by briefly staining the nitrocellulose membrane with Ponceau S. Following this, the nitrocellulose membrane was briefly washed two times in distilled water and incubated with 5% dry milk powder dissolved in Tris-buffered saline (TBS, pH 7.5) for 1 h at room temperature with gentle rocking. Subsequently, the nitrocellulose membrane was incubated with either lunasin, BBI or KTI antibodies that were diluted 1:10,000 in TBS containing 5% dry milk powder. Non-specific binding was eliminated by washing the membrane four times (10 min each wash) with TBS containing 0.05% Tween-20 (TBST). Specifically bound antibodies were detected by incubating the nitrocellulose membrane with 1:20,000 dilution of goat anti-rabbit IgG–horseradish peroxidase conjugate antibody (Bio-Rad) for 2 h. Following this the membrane was washed four times in TBST as above. Immunoreactive polypeptides were visualized by incubation of the membrane with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL).

2.10. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The three abundant proteins (21, 14 and 5 kDa) were excised from Coomassie stained SDS–PAGE gels, thoroughly washed in distilled water, and treated with 50% solution of acetonitrile containing 25 mM ammonium bicarbonate to destain the gel pieces. In-gel digestion of protein bands and MALDI-TOF-MS analysis of tryptic peptides were performed as described previously (Krishnan et al., 2009).

2.11. Realtime PCR analysis of interleukin (IL) gene expression

THP-1 human monocytes were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in 6-well plates (Costar; Corning) in RPMI 1640 media containing 10% fetal bovine serum and 1% amphotericin B/streptomycin/penicillin. The cells were incubated at 37 °C with 5% CO_2 in ambient air. Following overnight growth they were treated with 50 μM LPIC for two hours, the cell were then washed with PBS and resuspended in fresh medium containing 10 ng/mL of LPS. Two hours after LPS treatment the THP-1 cells were harvested by centrifugation at 400×*g* for 10 min and the resulting pellet was used for isolation of total RNA using the TRIzol method. Interleukin (IL-1β and IL-6) mRNA expression levels in THP-1 cells were examined by Realtime PCR. One μg of total RNA was used to reverse transcribe cDNA using StrataScript First Strand Complementary DNA Synthesis kit. Quantitation of mRNA levels was performed on ABI Prism 7900HT Sequence Detection System using TaqMan Universal PCR Master Mix (Huang, Fletcher, Niu, Wang, & Yu, 2012). Experiments were conducted in triplicate and data were reported as mean ± standard error. Significance differences between treatments were determined using one-way ANOVA and Tukey's Honestly Significant Difference (HSD) test.

3. Results and discussion

3.1. Optimization of extraction conditions

Aqueous ethanol has been previously used to purify protease inhibitors from soybean seeds (Frattali, 1969; Sessa & Wolf, 2001). For example BBIC, a serine protease inhibitor useful as a human cancer chemopreventive agent, is routinely isolated using aqueous ethanol (Kennedy et al., 1993). Recently, it was demonstrated that the BBIC also contains lunasin (Hsieh et al., 2010). However there has been no systematic analysis of the extraction conditions that is required for the enrichment of both lunasin and protease inhibitors. We therefore first examined the extraction efficiency of varying concentrations of aqueous ethanol. SDS–PAGE analysis of soybean proteins extracted with ethanol showed that the protein yield declined with increasing concentration of ethanol (Fig. 1A). In order to determine the ethanol concentration that provided the best yield of lunasin and protease inhibitor we employed Western blot analysis utilizing antibodies raised against chemically synthesized lunasin and Bowman Birk protease inhibitor. Lunasin antibodies strongly reacted against a 5 kDa protein (Fig. 1B). Additionally the antibody also reacted against 9 and 14 kDa proteins. Lower concentration of ethanol (0–40%) resulted in higher recovery of lunasin. Extraction of soybean seed powder with greater than 40% ethanol resulted in poor lunasin yield (Fig. 1B). The distribution of BBI in these ethanol-extracted samples was also examined by Western blot analysis. BBI-specific peptide antibodies recognized a protein with an apparent molecular weight of 14 kDa (Fig. 1C). Unlike lunasin, the yield of BBI was significantly lower in the absence of ethanol and increased gradually with increasing concentration of ethanol from 10% to50%. However, higher concentration of ethanol (greater than 50%) resulted in very low yield of BBI (Fig. 1C). We also examined the effect of varying the ratio of extraction solution (30% ethanol) to the amount of soybean seed powder. Altering the ratios over a range of 100:1–5:1 (30% ethanol:seed powder) indicated that the yield of lunasin and protease inhibitor was optimum at 10:1 ratio (data not shown). Based on these results, we chose 30% ethanol at 10:1 ratio for isolation of LPIC.

3.2. Enrichment of LPIC by calcium precipitation

Previously we have demonstrated that calcium precipitation can be utilized to preferentially isolate soybean seed storage proteins (Krishnan et al., 2009). We wanted to examine if the calcium precipitation procedure could also be utilized for the enrichment of lunasin and protease inhibitor from the 30% ethanol soybean extract. Consequently, we examined the effect of adding varying concentration of Ca^{2+} (0.5–20 mM) on the enrichment of LPIC. Calcium precipitated proteins were examined by SDS–PAGE (Fig. 2). The recovery of 5, 14 and 21 kDa proteins gradually increased with

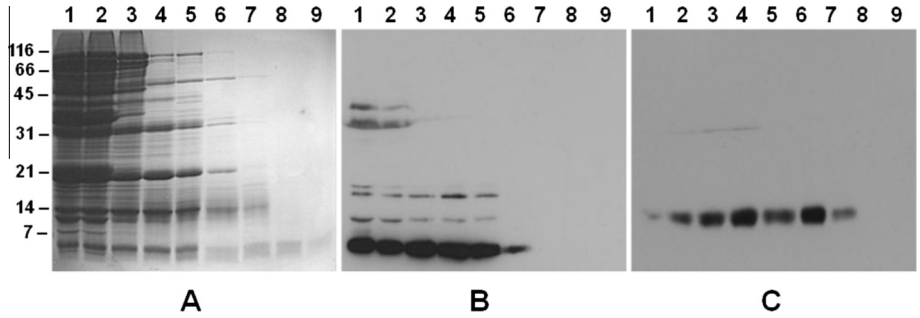

Fig. 1. Extraction of lunasin and protease inhibitors with aqueous ethanol. (A) Soybean cultivar Williams 82 seed flour was extracted with increasing concentration of ethanol and the extracted proteins were resolved by 15% SDS–PAGE. Resolved proteins were stained with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes and probed with lunasin antibody (B) or BBI peptide antibody (C). Lane 1, 0%; lane 2, 10%; lane 3, 20%; lane 4, 30%; lane 5, 40%; lane 6, 50%; lane 7, 60%; lane 8, 70%; lane 9, 80% ethanol extracted proteins. Molecular weight markers are shown on the left and designated in kDa.

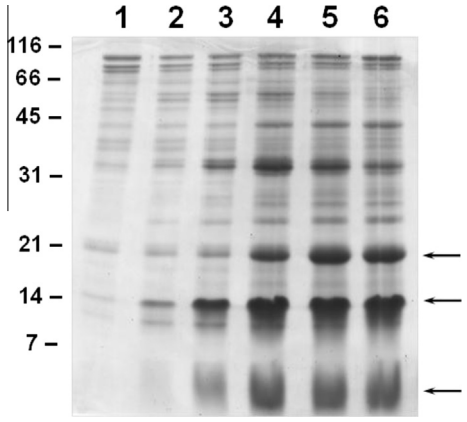

Fig. 2. Fractionation of 30% ethanol extracted soybean cultivar Williams 82 seed proteins with increasing Ca^{2+}. Calcium precipitated proteins were resolved by 15% SDS–PAGE and stained with Coomassie Brilliant Blue. Lane 1, 0.5 mM; lane 2, 1 mM; lane 3, 2 mM; lane 4, 5 mM; lane 5, 10 mM; lane 6, 20 mM calcium precipitated proteins. Molecular weight markers are shown on the left and designated in kDa. The arrows point to the abundant 21, 14 and 5 kDa proteins.

increasing concentration of calcium (Fig. 2). We excised these bands from the polyacrylamide gel and analyzed them by MALDI-TOF mass spectrometry resulting in the identification of the 21 kDa protein as Kunitz trypsin inhibitor, and the 14 and 5 kDa proteins as soybean 2S albumin precursor and lunasin, respectively (Table 1). The mass spectra of the peptide fragments generated from the tryptic digest of the 14 kDa protein, when searched against protein database entries revealed that they are similar to soybean 2S albumin precursor (Table 1). However, the reactivity of the lunasin antibody to the 14 kDa protein was less intense than the 5 kDa protein (Fig. 1B) indicating that the 14 kDa protein may represent the unprocessed 2S albumin precursor protein. It is known that lunasin, a 2S albumin protein, is synthesized as a precursor protein that is proteolytically processed onto a 5 kDa small subunit and 9 kDa large subunit. A recent study focused on large-scale purification of lunasin has demonstrated that the majority of the 5 kDa lunasin in soybean seed was present as a 14 kDa protein complex (Seber et al., 2012). It was shown that the 14 kDa lunasin complex is made up of the 5.1 kDa (lunasin) and 8.9 (large subunit of 2S albumin) peptides that are held together by means of two disulfide bonds. Thus, it appears that the 14 kDa protein seen in our LPIC preparation may represent the 14 kDa lunasin complex described by Seber and his associates (Seber et al., 2012).

3.3. Purification of LPIC by anion-exchange chromatography

To assist in obtaining large amounts of LPIC we scaled up our optimized small-scale purification procedure. By employing this procedure we were able to obtain 11.93 ± 0.25 g (wet weight) of LIPC from 100 g of soybean seed powder that was subsequently lyophilized yielding 3.24 ± 0.16 g of LIPC powder. To further purify LPIC we performed DE-52 anion-exchange chromatography. To facilitate large scale-purification, step-elution with increasing concentration of sodium chloride was performed (Fig. 3). Protein concentration from every alternate fraction was monitored by measuring the absorbance at 280 nm. SDS–PAGE analysis followed by Western blot analysis indicated that the three most abundant proteins all eluted from the DE-52 column between 300 and 400 mM NaCl (Fig. 3 insert). Even though anion-exchange chromatography resulted in elimination of few contaminating proteins from LPIC, this purification step did not substantially enrich LPIC. Furthermore, anion-exchange chromatography step lowered the yield of LPIC. Since our goal was to devise a method that was rapid and simple we concluded that the use of DE-52 anion-exchange chromatography was not an essential step for the preparation of LPIC.

SDS–PAGE analysis of lyophilized LPIC powder obtained by calcium precipitation and DE-52 anion-exchange chromatography revealed three abundant proteins with molecular weights of 21 kDa, 14 kDa, and 5 kDa, respectively (Fig. 4A). The LPIC also contained several other minor proteins (Fig. 4A). DE-52 anion-exchange chromatography step removed some of these contaminating proteins (Fig. 4A). Western blot results using antibodies specific for BBI, KTI and lunasin confirmed that these proteins are greatly enriched in our LPIC preparations (Fig. 4). Lunasin was barley detected from total soybean seed proteins while it could be readily detected in LPIC preparations demonstrating their presence in large quantity (Fig. 4D). To examine if the LPIC prepared by calcium precipitation method possesses protease inhibitor activity, we measured KTI and BBI activity using N-benzoyl-L-arginine ethyl ester and N-benzoyl-L-tyrosine ethyl ester as substrates, respectively. Both trypsin and chymotrypsin inhibitor activities were readily detected in the LPIC (data not shown). These observations in conjunction with the Western blot results demonstrate that the LPIC is enriched both in lunasin and enzymatically active protease inhibitors.

We also used seeds from soybean cultivar Maverick to examine if our rapid purification procedure to isolate LPIC can be applied to other soybean cultivars. When LPIC isolated from soybean cultivar Maverick was examined by SDS–PAGE the same three abundant proteins (5, 14, and 21 kDa) seen in soybean cultivar Williams 82 were also detected (Fig. 5A). The migration of the 5 kDa protein in the SDS–PAGE was identical to that of chemically synthesized lunasin (Fig. 5A). A densitometer scan of this gel indicated that the 5, 14, and 21 kDa proteins accounted for nearly about 60% and 80% of the total protein in LPIC and DE-52 anion-exchange chromatography purified fractions, respectively (Fig. 5B and C).

Table 1
MALDI-TOF-MS identification of the three abundant proteins in LPIC.

Band #	Protein identified (NCBInr accession)	MOWSE (50 ppm)	Sequence coverage (%)	Peptide matched
1	Kunitz trypsin inhibitor *Glycine max* (gi|3318877)	746	61	AAPTGNER CPLTVVQSR GIGTIISSPYR FIAEGHPLSLK IGENKDAMDGWFR DAMDGWFR VSDDEFNNYK LVFCPQQAEDDK LVFCPQQAEDDKCGDIGISIDHDDGTR CGDIGISIDHDDGTR RLVVSK NKPLVVQFQK LDKESL
2	2S albumin precursor; GM2S-1 *Glycine max* (gi|5902685)	340	50	WQHQQDSCRK GDDDDDDDDDNHILRTMR CCTEMSELRSPK IMENQSEELEEKQK MEKELINLATMCRFGPMIQCDLSSDD
3	2S albumin precursor *Glycine max* (gi|351727517)	150	15	QLQGVNLTPCEK IMENQSEELEEK

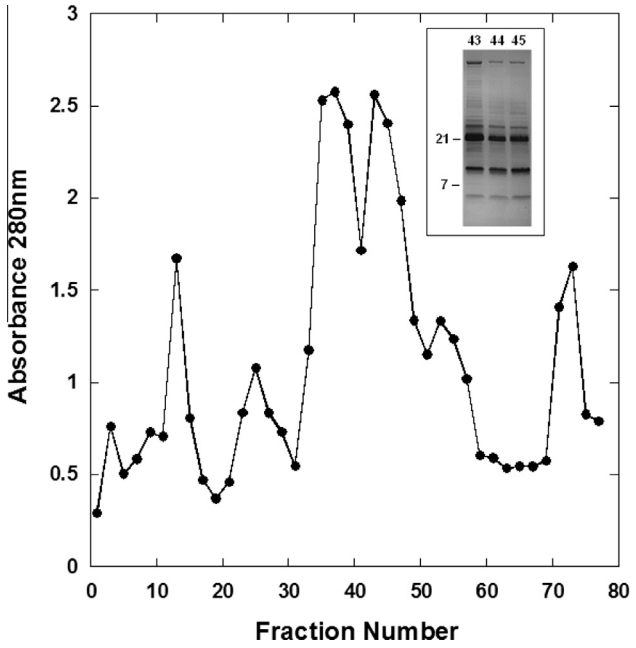

Fig. 3. Fractionation of LPIC by DE-52 anion-exchange chromatography. Calcium-precipitated proteins from 30% aqueous ethanol fraction were loaded on a DE-52 column and eluted with step sodium chloride gradient (50, 100, 200, 300, 350, 400, 500 and 1000 mM). Aliquots of each alternative fraction were resolved by 15% SDS–PAGE. The 21, 14 and 5 kDa proteins were enriched in fractions #43, #44 and #45 (insert).

3.4. LPIC inhibits lipopolysaccharide-stimulated cytokine expression

Lunasin plays an important role in preventing inflammation commonly associated with the development of chronic diseases (de Mejia & Dia, 2009). Overproduction of pro-inflammatory cytokines is a general response to extended, unregulated immune response (Coussens & Werb, 2002). To examine if LPIC can inhibit overproduction of pro-inflammatory cytokines we took advantage of *in vitro* cell culture assay based on lipopolysaccharide-stimulated cytokine expression in THP-1 human monocyte lines (Fig. 6). Earlier we had optimized the conditions required for LPS-induced changes in cytokine mRNA expression in THP-1 cells (Huang et al., 2012). The expression of IL-1β and IL-6 in THP-1 cells in the presence and absence of LPIC (50 μM) is shown in Fig. 6. As expected LPS stimulated the expression of both IL-1β and IL-6 but cells pre-treated with LPIC exhibited drastic reduction of the LPS-induced expression of these two cytokine mRNA levels (Fig. 6).

Bowman Birk protease inhibitor is known to be effective suppressors of carcinogenesis in *in vitro* and *in vivo* model systems (Armstrong et al., 2000, 2003; Kennedy, 1998; Kennedy & Wan, 2002; Yavelow et al., 1985). These studies have now established that BBI enriched soy fraction (BBIC) is equally effective as the highly purified BBI as a cancer chemopreventive agent. BBIC is now commonly used both in animal models as well as in clinical trails in cancer patients. Analysis of the protein composition of the BBIC has shown that lunasin and BBI are present at concentrations of 360 and 74.4 ng/μg protein, respectively (Hsieh et al., 2010). The two bioactive peptides account for 44% of total protein of the BBIC. This study also reported that lunasin, not the BBI, as the bioactive cancer preventive agent in BBIC. The authors suggested that the role of BBI in the BBIC is simply to protect lunasin from digestion when soybean protein is consumed by humans (Hsieh et al., 2010). Based on this study it appears treatment of cancer patients with soy protein concentrate such as LPIC may be much more efficacious than administering purified lunasin and BBI alone separately.

The LPIC reported in our study contains significant amounts of protease inhibitors. Unlike the BBIC, the LPIC contains high amounts of Kunitz trypsin inhibitor. It is likely that KTI may have a similar role as BBI in protecting the lunasin. An earlier study has demonstrated that both BBI and soy Kunitz Trypsin Inhibitor can protect lunasin from *in vitro* digestion with pancreatin (Hsieh et al., 2010). In addition to KTI the LPIC also contains significant amounts of BBI. The Western blot results using antibody specific to soybean BBI clearly reveal their presence in the LPIC. Additionally, LPIC also exhibits high levels of chymotrypsin inhibitor activity. Importantly, our LPIC preparation shows biological activity as evidenced by its ability to suppress the expression of pro-inflammatory cytokines. The fact that the LPIC contains high concentration of protease inhibitors and lunasin, two soy peptides with known anticancer properties, argues well for the use of LPIC in animal and human clinical studies.

In this study we describe a rapid, simple and an inexpensive method for obtaining a protein fraction that is highly enriched in lunasin and protease inhibitors. Unlike some previous studies whose aim was to obtained large amounts of highly purified

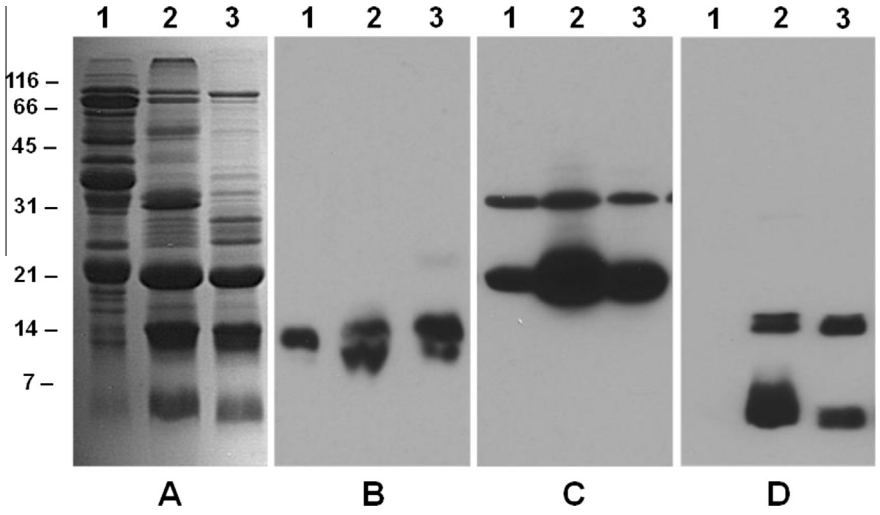

Fig. 4. SDS–PAGE analysis of Lunasin Protease Inhibitor Concentrate (LPIC). Panel (A). Calcium precipitated proteins (lane 2) were fractionated by DE-52 anion-exchange chromatography. Proteins eluting between 300 and 350 mM sodium chloride were pooled and an aliquot was resolved by SDS–PAGE (lane 3). Soybean seed total protein (lane 1) is also included for comparison. Proteins seen in Panel (A) were transferred to nitrocellulose membranes and probed with BBI peptide antibody (Panel B) or KTI antibody (Panel C) or lunasin antibody (D). Molecular weight markers are shown on the left and designated in kDa.

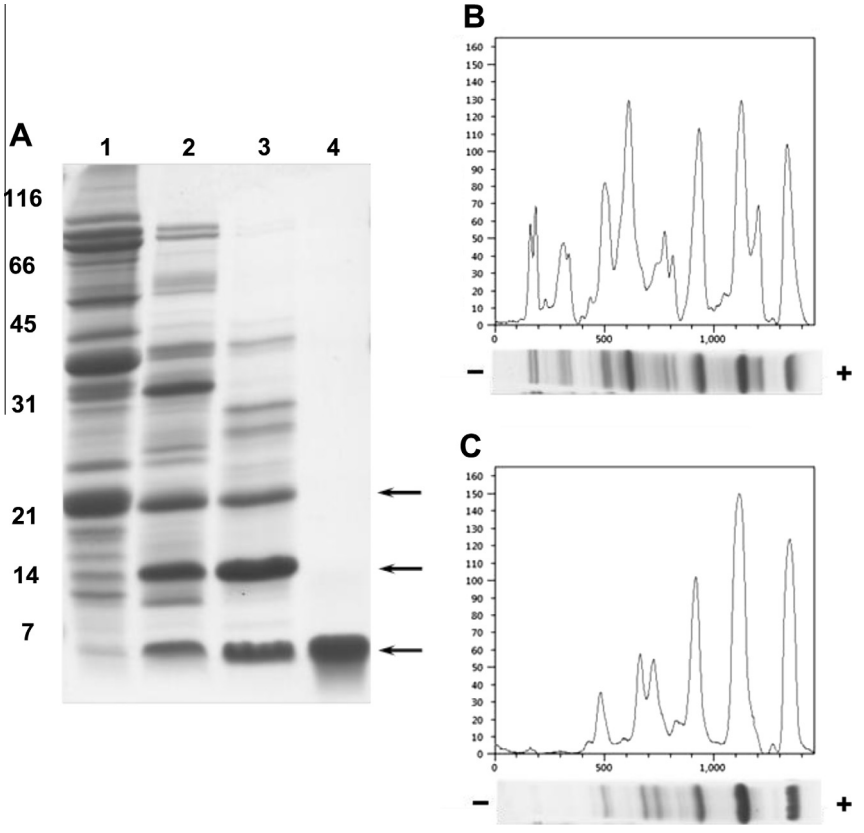

Fig. 5. SDS–PAGE analysis of Lunasin Protease Inhibitor Concentrate (LPIC). Panel (A). LPIC was resolved by 15% SDS–PAGE and stained with Coomassie Brilliant Blue. Molecular weight markers are shown on the left and designated in kDa. Lane 1, total seed protein; lane 2, calcium precipitated proteins; lane 3, DE-52 anion-exchange chromatography eluted fraction and lane 4 chemically synthesized lunasin. Quantification of the band intensity in lanes 2 (Panel B) and 3 (Panel C) was determined by densitometry utilizing Phoretix 1D gel analysis software.

lunasin, our focus was to obtain a biologically active lunasin enriched preparation. Our simple cost-effective procedure avoids the use of time-consuming anion-exchange chromatography and size-exclusion chromatography and the entire isolation procedure can be completed in less than 2 h. Since LPIC isolated by our novel method shows biological activity it can be used for large-scale animal and human trails. Our method can be easily scaled up to yield kilograms of highly enriched lunasin preparation. Furthermore, to obtain a highly purified preparation of lunasin for therapeutics, one can use our LPIC as a starting material for further purification.

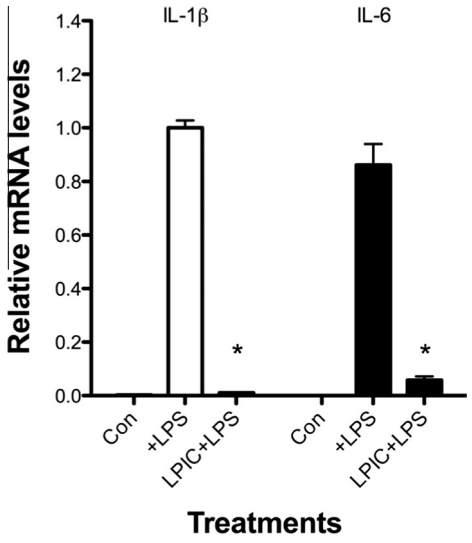

Fig. 6. LPS induction of cytokine mRNA expression in THP-1 monocyte cells is suppressed by LPIC. THP-1 cells were treated in presence or absence of LPIC for 2 h and an additional 2 h induction by LPS as described in Section 2. Real-time PCR analysis revealed that the addition of LPIC down-regulated the LPS-induced expression of IL-1β and IL-6. Each bar represents the mean ± SEM ($n = 3$).

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